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GENETIC STRATEGIES FOR STUDYING BACTERIAL DIFFERENTIATION

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I. INTRODUCTION

In this article I attempt to find and discuss common features of genetic analysis of cellular differentiation in bacteria. By cellular differentiation is meant formation of one distinguishable cell type from another; formation of bacterial organelles is not considered. By genetic analysis is meant the analysis of the organization and control of expression of genes necessary for differentiation. Bacteria from many species differentiate, but few of these systems have been studied to any extent by genetic means. The four main examples are listed in Table 1. I have restricted myself to selected topics and

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Table 1. *Differentiating bacteria that have been analysed genetically*

Species	Differentiation	Reference
Bacillus subtilis	Endospore formation, germination	Piggot & Coote, 1976 Smith, Moir & Sammons, 1978
Streptomyces coelicolor	Formation of aerial mycelia, spore formation in hyphae	Chater & Hopwood, 1973 Kalakoutskii & Agre, 1976
Myxococcus xanthus	Swarming, aggregation, fruiting body formation: myxospore formation	Wireman & Dworkin, 1975
Caulobacter crescentus	Cell cycle	Shapiro, 1976

use selected examples for illustration. The article is on bacterial differentiation, but it should be mentioned at the outset that very similar types of analysis have been described for eukaryotes, notably *Saccharomyces cerevisiae* (Hartwell, 1974, 1978; Baker *et al.*, 1976), and for bacteriophage (Rabussay & Geiduschek, 1977).

The four bacterial species differ considerably in the morphology of their differentiation. Thus a common genetic approach to their study need not necessarily lead to common conclusions. In three of the species the cell differentiation is a consequence of starvation and each results in a resting cell (or spore), albeit in very different locations – inside a mother cell, at the end of an aerial hypha and inside a multi-cellular fruiting body. These can be thought of as terminal differentiations. In each case the bacteria can grow vegetatively without differentiation, and it is possible to have mutants that cannot differentiate under any circumstance. In *Caulobacter crescentus*, however, the cell cycle itself is considered to be the differentiation process, so that the mutants studied must allow the differentiation to proceed under some conditions.

It is a matter of semantics whether the vegetative cell cycle is considered a type of differentiation. It is often held to be so where division of the cell results in two distinct cell types as with *C. crescentus*, and this is the view taken here. In any case genetic analysis of the cell cycle in *C. crescentus* (and in *S. cerevisiae*) has proceeded in similar directions to the analysis of the different types of terminal differentiation.

Where vegetative cell division results in two (apparently) identical daughter cells, notably in *Escherichia coli* and *Bacillus subtilis*, it seems more dubious to consider the process as differentiation. Moreover it has not been possible to draw information together in the way that is attempted for the systems listed in Table 1, and I shall not be considering division of *E. coli* or *B. subtilis* further. Nevertheless considerable information is available, and two recent reviews have included authoritative discussion of genetic aspects of growth and division of these species (Slater & Schaechter, 1974; Sargent, 1979).

The species I have indicated as being my prime concern are all ones in which efficient systems of genetic exchange between bacteria are available. However, considerable 'genetic' information can be gleaned from a study of mutants without the need for genetic exchange. I will consider first the use of mutants *per se* and then consider the additional methods that require genetic exchange.

The article is confined, in the main, to processes that appear to be specific for differentiation. Mutation in such a process would affect differentiation but not other aspects of cell metabolism. This restriction removes, for example, energy production from consideration. Such features as energy production are clearly essential to differentiation and will need to be incorporated into the complete picture of differentiation. However, the picture is at present very incomplete, and it is thought that their omission here will improve the clarity of an attempt to assemble the pieces of one part of the picture.

II. GENETICS WITHOUT GENETIC EXCHANGE

It is taken as self-evident that much genetic information can be gained without genetic exchange and without mutants – by studying differentiation in the appropriate wild-type, and by comparative studies of differentiation in related species. In this section, the emphasis is on the information that can be, and has been, gained by the study of mutants. Generally the mutants considered are those that are blocked in differentiation, such as *spo* mutants of *B. subtilis*. Such mutants are usually affected in a range of characters associated with differentiation. Consequently it is difficult to identify the primary lesion, and in most cases this has not been achieved.

(i) *Mutants that may have multiple lesions*

It must be assumed that a mutant could have more than one mutation until the contrary is established. Some information can be gained from mutants with multiple lesions. This information can clearly also be gained when the mutants are known to have single-point mutations; the examples quoted are not intended to imply that the mutations involved are multiple, but merely that the conclusions discussed here do not require single mutations. The analysis is, essentially, a special case of comparative morphology or biochemistry. For example, some asporogenous mutants of *B. subtilis* have coat but not cortex around the prespore, whereas others have cortex but not coat (Piggot & Coote, 1976). Thus deposition of coat around the prespore need not depend on prior deposition of cortex nor vice versa. Exactly the same conclusion can be reached from comparative morphology: *B. subtilis* forms cortex before coat deposition (Ryter, 1965), whereas certain *Clostridia* deposit coat around the prespore before they lay down cortex (Mackey & Morris, 1971; Rousseau, Hermier & Bergere, 1971).

Information can also be gained about the relevance of morphological and biochemical events to the differentiation. Thus, in *C. crescentus* wild-type, the cell cycle includes the appearance of flagella and of a stalk structure. Some mutants have been isolated that lack flagella and others that lack the stalk structure; both types are otherwise unaffected in their cell cycle and so the structures are unnecessary for the cell cycle (Marino, Ammer & Shapiro, 1976; Osley & Newton, 1977). In like manner biochemical events can be removed from consideration. *B. subtilis* synthesizes large amounts of α-amylase at the start of spore formation; mutants are known that lack α-amylase and sporulate normally so that excretion of α-amylase is not necessary for spore forma-

tion (Schaeffer, 1969). Unfortunaetly the argument is not always clear-cut for biochemical events, as 'negative' mutant depends on a quantitative rather than a qualitative judgement. This reservation is central to the controversy surrounding the role of antibiotic in endospore formation: mutants are known that sporulate and do not make detectable antibiotic, but this could still leave a thousand, or more, molecules of antibiotic for each bacterium (reviewed Sadoff, 1972; Katz & Demain, 1977).

(ii) *Mutants that have single-point mutations*

In species allowing genetic exchange, the strains studied have usually been constructed by such exchange. A small piece of chromosome, harbouring the mutation in question, is transferred from the original mutant isolate into a standard strain such as the wild-type. The constructed strain is said to be isogenic with the standard strain except for the mutation in question. The two strains can then be compared and the difference between them presumed to result from a single mutation. This bland presumption is questionable for several reasons. Firstly the transferred DNA might carry two or more mutations. This is particularly likely to be so when nitrosoguanidine has been used for mutagenesis, as it frequently causes closely linked multiple mutations (Guerola, Ingraham & Cereda-Olmedo, 1971). Secondly, the mutation could be a deletion affecting two or more genes. Thirdly, two pieces of donor DNA could have been introduced during the cross. Fourthly, the genetic cross itself might possibly have introduced new mutations (Yoshikawa, 1966). Each of these possibilities, although remote, should be guarded against. However, when the lesion in question repeatedly behaves as a single-point mutation, it becomes increasingly likely that it is so.

An alternative to the above is a careful study of reversion of the mutant to the wild-type. This can provide a more rigorous demonstration that the original lesion was a single-point mutation. It does not require a system of genetic exchange. It provides a pair of strains, the mutant and the revertant, that are truly isogenic except for the mutation in question.

The necessity for establishing that the lesion is a single-point mutation is that many of the mutations blocking differentiation are pleiotropic (i.e. affect more than one characteristic in the phenotype). In a series of mutants this pleiotropy is found to be ordered as in spore formation of *B. subtilis* (Balassa, 1969; Waites *et al.*, 1970). It is illustrated with data from Waites *et al.* (1970) (Table 2), when the order of characters are: protease ⟶ alkaline phosphatase ⟶ glucose dehydrogenase ⟶ dipicolinic acid ⟶ heat resistance. Asporogenous mutants that are deficient for one of these characters are always also deficient for the characters shown to its right. Ordered pleiotropy has also been found in spore formation of *Streptomyces coelicolor* (Hopwood, Chater, Dowding & Vivian, 1973), in fruiting-body formation of the eukaryote *Dictyostelium discoidium* (Loomis, Morrisey & Lee, 1978), in the cell cycle of *C. crescentus* (Osley & Newton, 1977) and of the eukaryote *S. cerevisiae* (Hartwell, 1974). In each case it was taken to indicate a dependent sequence of events in which occurrence of late events is dependent on the correct occurrence of all earlier events in the sequence.

Table 2. *Sporulation phenotypes resulting from asporogenous mutations in* Bacillus subtilis (*based on Waites* et al., 1970)

(In the wild-type Pr is associated with stage O, AP with stage II, GDH with stage III, DPA with stage V and HR with stage VI. Abbreviations used: Pr, protease; AP, alkaline phosphatase; GDH, glucose dehydrogenase; DPA, dipicolinic acid; HR, heat resistance. Stages of sporulation are as defined by Ryter (1965): O, vegetative bacterium; II, spore septum formed; III, prespore free within mother cell; IV, prespore has primordial germ cell wall and/or cortex; V, spore coat formed. Numerals indicate the order of occurrence in *B. subtilis*. Genetic loci are as defined by Piggot & Coote (1976).)

	Sporulation phenotype					
Mutant locus	Stage	Pr	AP	GDH	DPA	HR
spoOA	O	−	−	−	−	−
spoIIA	II	+	−	−	−	−
spoIIIE	III	+	+	−	−	−
spoIVF	IV	+	+	+	−	−
spoVD	V	+	+	+	+	−
(spo$^+$)	VII	+	+	+	+	+

The studies cited necessarily concerned only a few of the genes involved, and so only gave a general idea of the differentiation process. There are indications that more than one dependent sequence might be operating (Coote & Mandelstam, 1973; Hartwell, 1974, 1978; Piggot, 1978). Moreover the system could in principle be controlled at transcription of DNA into RNA, at translation of RNA into protein, at assembly of structures, or at any combination of these. In bacterial endospore formation, there is clear evidence for continuing synthesis of new species of mRNA (Doi, 1977), so that transcriptional control is involved. For this, sequential induction of operons provides a useful working model (Halvorson, 1965). There is also suggestive evidence for translational and post-translational controls (see Piggot & Coote, 1976; Sonenshein & Campbell, 1978). When the final picture emerges for the control of spore formation, it is likely to prove complex. It should be remembered that there is already known to be a sophisticated system of transcriptional and post-transcriptional control in the development of bacteriophage (Eisen, 1975; Kellenberger, 1976; Rabussay & Geiduschek, 1977).

As discussed earlier, the nature of the primary lesion is not usually known in differentiation mutants. What is seen in such mutants is the end, or terminal, phenotype. Because of the complexity of the process, the time of manifestation of this end phenotype may be considerably removed from the time of the initial effect of the genetic lesion. Moreover, the order of mutations inferred from the end phenotype need not be the order of transcription of the corresponding genes.

(iii) *Conditional mutants*

Many mutants exhibit the mutant phenotype in one set of conditions, termed restrictive or non-permissive, and are indistinguishable from the wild-type in another,

termed permissive. Such conditional mutants are almost the only ones available for studies of the cell cycle, as absolute, or unconditional, mutants would generally be inviable. This is not so for processes of terminal differentiation where absolute differentiation-defective mutants are still viable. In this section, experiments that take advantage of the conditional nature of the mutants are discussed.

The most common type of conditional mutant has high temperature as the restrictive condition, and low temperature as the permissive condition. All the examples concern these temperature-sensitive mutants. However, the methods can often be adapted to other types of conditional mutant. Temperature-sensitive mutants are, by their nature, likely to be single-point mutations. Nevertheless, exceptions exist (e.g. *S. cerevisiae cdc 22*; Hartwell, 1974), and some care should be exercised to avoid unwarranted deductions.

There are two basic experiments. In the first, differentiation starts at the permissive temperature and, at different times thereafter, cultures are transferred (shifted-up) to the non-permissive temperature. This gives the time in the process at which the mutant becomes insensitive to the non-permissive temperature (sometimes called the execution point). The second experiment is the converse in which cultures are maintained initially at the non-permissive temperature and transferred at different times to the permissive temperature (often called the shift-down experiment). The second experiment has been used in studies of terminal differentiation, but will not readily be applicable to analysis of the cell cycle until there is good cell synchrony.

Most temperature-sensitive mutants are thought to be defective in a protein that has been rendered thermolabile (although there are exceptions where tRNA molecules have become thermolabile; Gallucci, Pacchetti & Zangrossi, 1970; Smith, Barnett, Brenner & Russell, 1970). The protein may be temperature-sensitive during its synthesis and/or when it is fully formed; the heat inactivation may or may not be reversible. Consequently there is some ambiguity in interpreting the results of temperature-shift experiments. Nevertheless, the immediate gene product is being affected so that studies identifying the temperature-sensitive period during differentiation give a more accurate estimate of the time of gene expression than do studies identifying the terminal phenotype of a mutant.

The most extensively studied bacterial system is sporulation of *B. subtilis* (reviewed by Piggot & Coote, 1976) and the fullest analysis of particular mutants is by Young (1976) (for analogous studies with *S. cerevisiae*, see Esposito, Esposito, Arnaud & Halvorson, 1970). Young added to the two basic experiments by incubating cultures at the non-permissive temperature and giving them pulses (at the start of the temperature-sensitive period), of varying times at the permissive temperature.

Two main general conclusions have emerged. The first is that different loci are expressed for different lengths of time: some *spo* loci needed only to be expressed for as little as 15 min whilst others had to be expressed for several hours. This conclusion may seem obvious in retrospect. However, so little is known about the 35 *spo* loci of *B. subtilis*, for example, that it is very easy to overgeneralize and assume that all loci are expressed in the same way. If each locus has distinctive features, it will have to be considered separately, and models of the control of development will have to deal with

the foibles of each locus. Hypothetical models dealing with idealized loci must be regarded as distant preliminaries to the definitive final model.

The second main conclusion came as a surprise, at least to the author, but was true for most temperature-sensitive *spo* mutants of *B. subtilis*. It was that temperature-sensitive mutants could not idle at the non-permissive temperature. Thus in shift-down experiments, when mutants were maintained in non-permissive conditions for some time after the start of the sensitive period, sporulation was not merely delayed, it was prevented. The most extreme case was noted by Young (1976) who found one mutant for which as little as 15 min at the non-permissive temperature in the sensitive period would prevent sporulation. This behaviour indicates a rather delicate control for the process. It obstructs reciprocal shift experiments with double mutants (see later). It would make cross-feeding of one mutant by another rather harder to accomplish.

The temperature-sensitive nature of the mutants offers the faint hope that the particular protein involved, and hence the primary lesion, can be identified. To have much hope of success, this would require a series of suitable candidate proteins for testing. Usually there are few such candidates. Moreover, to be unambiguous, it requires that pure proteins from wild-type and mutant can be compared in exactly similar conditions. Failure to do this leaves the possibility that some other component of the mutant cytoplasm than the particular protein being considered is different from the wild-type, and that this component could affect the thermostability of the protein; thus the particular protein could appear temperature-sensitive in the mutant and yet not be the product of the mutant gene.

(iv) *Initiation mutants*

In certain systems it may be possible to identify or even select mutants that are altered specifically in the initial stages of differentiation. The precise method may depend on the species used, and the methods for terminal differentiation are likely to be different from those for the bacterial cell cycle, where it is even debatable whether there is a particular start to the cycle.

For investigating terminal differentiation two methods have been applied to endospore formation that might have more general applicability. Michel, Cami & Schaeffer (1968) described a method for isolating mutants of *B. subtilis* that were unable to start sporulation. In certain relatively poor media these bacteria may grow, but a significant proportion of the population stop growing and start to sporulate. In the same medium the whole population of a mutant, unable to initiate sporulation, may continue to grow and so the mutant has, potentially, a selective advantage over the wild-type. Applying this rationale, Michel *et al.* (1968) isolated, without prior mutagenesis, a preponderance of mutants blocked in the *spoOA* locus. By various other criteria this locus is also associated with the initial steps of sporulation.

The other approach uses the fact that the differentiated cell, the spore, is more resistant than the vegetative cell to such a treatment as heating. Consequently it is possible to isolate mutants that sporulate in conditions in which the wild-type would

not. Various types of such derepressed mutants have been isolated in *Bacilli* (reviewed by Piggot & Coote, 1976).

Each of the arguments in the preceding paragraphs could be taken to imply that there is a single initiating event for differentiation. However, this is not necessarily so. Initiation could well involve several concurrent events. In this case the various initiation mutants would presumably be altered in one of those events.

(v) *Mutants with a defined lesion*

A number of mutations causing defined lesions have been found to affect development. Such mutations can affect cell constituents, e.g. the ribosomes, or they can affect metabolic paths. It is well established that cell functions, such as RNA synthesis, protein synthesis, and energy generation, are required for differentiation. Unfortunately it has not as yet been possible to draw much more precise conclusions than this in relation to the present discussion. In particular, it is still not clear if these various functions have specific roles in controlling gene expression during differentiation, or if their effects are indirect. The defined lesions generally affect other cell functions besides those of differentiation and they are not considered further. Detailed consideration of these matters, in relation to endospore formation, has been given by Doi (1977), Freese (1972), and Sonenshein & Campbell (1978).

Mutations affecting DNA synthesis are somewhat different. Synthesis of DNA is an integral part of the cell cycle, so that DNA-synthesis mutants are cell-cycle mutants (Hartwell, 1974, 1978; Shapiro, 1976). Synthesis of DNA is also tied firmly to endospore formation since bacteria prevented from completing rounds of DNA replication are blocked at a very early stage of spore formation (Mandelstam, 1976). In some instances the nature of the DNA-synthesis mutation is known. However, it is again not yet possible to relate control of DNA synthesis to other aspects of cell differentiation in any precise way.

III. GENETICS WITH GENETIC EXCHANGE

The 'classic' systems of genetic exchange in bacteria, transformation, conjugation and transduction, have been limited to relatively few species of differentiating bacteria. This has led to a concentration of genetic studies on those few species, although, as argued in the previous section, considerable genetic information can be gained without the need for genetic exchange. The number of species amenable to these types of genetic exchange is gradually expanding. Of particular interest are the reports of transformation of the cyst-former *Azotobacter vinelandii* (Page & Sadoff, 1976) and of genetic exchange amongst various cyanobacteria (Ladha & Kumar, 1978) a group that includes some of the most sophisticated examples of prokaryotic differentiation (Carr & Bradley, 1973; Stanier & Cohen-Bazire, 1977).

In the sections that follow some uses to which classic genetic exchange can be put will be discussed. Before doing this, mention should be made of the 'new' systems of genetic exchange, genetic engineering and cell fusion.

Genetic engineering and its potential uses have been widely discussed and I do not propose to consider them at length here. One such potentiality is to make possible efficient dominance and complementation tests and to facilitate detailed fine-structure analysis through the production of many distinct mutations in cloned fragments of DNA. This all reinforces the 'classic' genetic methods, and should be most effective where the species studied is itself the host for cloning. The novel part of this potential use is to permit identification of specific genes, and specific mRNA and protein products from those genes. It should then be possible to determine the times of appearance of these gene products during differentiation. Presumably tests of the control of gene function should also be possible. Where the function of the original gene is unknown, cloning will allow identification of the mRNA and protein products of the gene (if any), and it will be the function of these products that is unknown. However, this may not be an insuperable barrier, given the increasing power of the techniques of molecular biology. The other possible major barrier to the approach is the uncertainty as to whether cloned differentiation genes will be expressed at all.

Fusion of bacterial protoplasts has now been described for *Bacillus megaterium* (Fodor & Alföldi, 1976), for *B. subtilis* (Schaeffer, Cami & Hotchkiss, 1976) and for several species of *Streptomyces* (Hopwood, Wright, Bibb & Cohen, 1977; Hopwood & Wright, 1978; Baltz, 1978). Bacterial protoplasts can be fused by the action of an agent such as polyethylene glycol. The cell wall is then caused to regenerate so as to give back normal bacteria, a process sometimes described as reversion, but not to be confused with the use of the word by geneticists as reversion of a mutation. There is no evidence for any extensive growth as protoplasts nor of extensive growth as a diploid. The end result of the fusion and wall-regeneration is haploid recombinants. The frequency of recombination between pairs of genetic markers is high, but is reduced when the markers are closely linked (Schaeffer *et al.*, 1976), so that it should be possible to construct linkage maps from frequencies of recombination. As yet it is not possible to say how fine or coarse the detail of such a linkage map will be. Recombination following fusion has already been demonstrated for several species for which no other form of genetic exchange is known, and it may be possible to extend the method to many other such species. So far fusion has only been reported for Gram-positive bacteria. The surface structure of Gram-negative bacteria is very different and it will be interesting to see if they can also be fused.

For Gram-positive bacteria the most exacting element in the technique, appears to be the regeneration of walled bacteria from protoplasts (reviewed by Landman, De Castro-Costa & Bond, 1977; Ward, 1978). This process has been found to be somewhat temperamental, and it may be that some strains are more suitable than others.

(i) *Construction of a genetic map*

The bread and butter of genetic exchange is the construction of a genetic map. This provides considerable information in itself and sets the scene for further studies utilizing genetic exchange. There are now extensive mapping data for sporulation in

B. subtilis (Piggot & Coote, 1976) and in *Streptomyces coelicolor* (Chater, 1972; Merrick, 1976). These maps give a minimum estimate of the number of loci in the process being studied. Reasonable estimates of the number of loci can nevertheless be obtained from a series of genetic crosses without the need for an extensive map (see Hodgkin & Kaiser, 1977). With a minimum estimate of the number of loci, it is possible to make a statistical estimate of the total number, by measuring the probability of new mutations falling in known loci; for *B. subtilis* the total number of *spo* loci was estimated to be 42 with 68% confidence limits of 33 and 59 (Hranueli, Piggot & Mandelstam, 1974). Such an estimate is clearly crude, but it gives an idea of the complexity of the situation. It is likely that transcription of many of the loci is specifically switched on during differentiation. As many of the loci are well separated on the genetic map, this implies that each has its own switch-on element in addition to associated structural genes. Thus the loci can be thought of as distinct operons (for detailed discussion, see Piggot, 1973). Sporulation of *B. subtilis* involves, therefore, control of the expression of some 40 to 50 *spo* operons (quite apart from any other controls).

(ii) *Studies with (mero) diploids*

Studies of diploids are often held to be of prime importance in genetic analysis. Thus the dominance test and the complementation test were critical to the understanding of the *lac* operon (although, as pointed out by Beckwith & Rossow, 1974, a degree of serendipity was also required in the analysis). These tests have not yet been extensively used for studies of bacterial differentiation, and it is interesting to explore why not. Partly it reflects the paucity of systems giving diploids, or merodiploids, that are stable throughout the differentiation. This difficulty may perhaps be overcome by suitable cloning systems. However, the other problem is that interpretation is not as easy as it might at first appear. Analysis of diploids is extremely useful when combined with fine-structure genetic analysis and a detailed knowledge of the immediate products of gene expression. One or both of these last two features is generally lacking in differentiating systems, and this severely restricts interpretation.

In the first instance analysis of a diploid containing both mutant and wild-type alleles of a gene should indicate if the mutant allele is dominant or recessive to the wild-type. Dominance precludes complementation tests and suggests that the mutation in question might lie in some kind of regulatory region. However, it is also possible that the mutation is in a gene coding for a protein subunit of some multimeric structure. More information is required to distinguish between these totally different possibilities. Without this, knowledge that a mutant allele is dominant to the wild-type remains essentially an isolated, if tantalizing, fact from which no definite conclusions can be drawn. Again it is to be hoped that cloning will let us out of this impasse.

The complementation test is possible with two mutant alleles that are recessive to the wild-type. At first sight it should give a method for estimating the number of genes (or cistrons) involved in differentiation. Where the test is easy, the method may be faster than the alternative of mapping. The problem is again that the immediate gene (or cistron) product is not known. Complementation if measured by the ability of the

(mero) diploid to differentiate is unlikely to be related quantitatively to the immediate gene product. This confuses interpretation, and it will not be possible to distinguish inter- from intra-cistronic complementation without further information. The distinction can be made with the aid of genetic mapping and an estimate made of the number of genes, but only to the resolution that the mapping itself would have given. The complementation test becomes much more valuable for analysing small genetic regions of which a fine structure map is available.

When two recessive alleles do not complement each other, it is strong evidence that the mutations lie in the same gene. This conclusion is not subject to the reservations that affect positive complementation, although there must be some slight reservation if it is not possible to determine the phenotype of a diploid in which one copy of the DNA is wild-type and the other copy carries the two mutations (Lewis, 1951).

(iii) *Ordering of two steps in a sequence*

In a sequence including events A and B, the problem arises how does A relate to B? Does occurrence of B depend on previous occurrence of A, or does occurrence of A depend on previous occurrence of B; do they occur independently of each other, or do they occur together (interdependently)? In the sequence of events during bacterial differentiation it is often possible to distinguish between these possibilities. Different methods have been used and they involve studying two blocks to differentiation. The two main types of method, using constructed double mutants, and using the reciprocal shift, can now be considered.

(*a*) *Constructed double mutants.* In this method a strain is constructed bearing two mutations that, taken singly give rise to distinguishable blocks in the differentiation process. The phenotype of the double mutant is then compared with those of the two strains that have only one of the mutations. This has been done for sporulation of *B. subtilis* (Coote & Mandelstam, 1973) and of *S. coelicolor* (Chater, 1975), and also for the cell cycle of *S. cerevisiae* (Hereford & Hartwell, 1974). Features of the method are illustrated with some of the results of Coote & Mandelstam (Table 3). The first three double mutants have the same phenotype as one of the parental strains, so that there is a clear epistatic relationship consistent with a dependent sequence of gene expression *spoOA* ⟶ *spoIIE* ⟶ *spoIVF*. It should be noted that this does not establish that there is such a dependent sequence. Chater (1975) is even more cautious in interpreting a similar epistatic relationship for *S. coelicolor*. The last double mutant (Table 3) has a phenotype that is clearly different from either parent. This indicates that both mutations are expressed in the double mutant and therefore that expression of each locus is independent of the other.

(*b*) *Reciprocal shift.* A group of methods, collectively called reciprocal shift, have been introduced in studies of bacteriophage development (Jarvik & Botstein, 1973) and of the yeast cell cycle (Hereford & Hartwell, 1974). They are applicable in principle to bacterial differentiation and so are considered here. The methods employ two treatments that block the process specifically and can be imposed independently and reversibly. The pairs of blocks can consist of a hot-sensitive (hs) and a cold-sensitive

Table 3. *Comparison of phenotypes of single and constructed double mutants (based on Coote & Mandelstam, 1973)*

Relevant genotype	Phenotype
spoOA147	O
spoIIE172	II
spoIVF152	IV
spoVD156	V
spoOA147 spoIIE172	O
spoOA147 spoIVF152	O
spoIIE172 spoIVF152	II
spoIVF152 spoVD156	III

The numbering of the stages (Ryter, 1965) gives the order of their occurrence in *Bacillus subtilis* (see Table 2).

(cs) mutation; a temperature-sensitive mutation and sensitivity to an inhibitor; or sensitivity to two different inhibitors. When hs and cs mutations are used, these are combined to form a double mutant. Two experiments are performed with the double mutant. In the first, the double mutant is allowed to develop at the high temperature. After the sensitive period for both lesions has elapsed, the mutant is then shifted to the low temperature and development allowed to continue. The second experiment is the reciprocal of the first with the shift being from low to high temperature. Depending on whether development is completed in both, neither or either experiments, it is possible to say if the mutated genes act independently, interdependently, or in a particular dependent sequence (for detailed discussion, see Jarvik & Botstein, 1973; Hereford & Hartwell, 1974). Any order deduced is unambiguous and does not depend on the precision of the phenotypic characterization of the blocks and indeed they can be indistinguishable phenotypically, so that the method has advantages over the constructed double-mutant method. The main limitation is the dearth of suitable reversible blocks. Moreover, the results could be misleading if a block is not truly reversible. Where the block is not reversible and the reciprocal shift cannot be performed, the use of drug sensitivity becomes an extension of the method for constructed double mutants.

(iv) *Suppressors*

(*a*) *Nonsense suppressors*. Nonsense mutations cause the release of incomplete proteins from ribosomes. Nonsense suppressors correct the defect and allow mature protein to be synthesized. Nonsense suppressors have been extensively analysed in *E. coli* and have been shown to be mutant tRNAs (Gorini, 1970). Similar suppressors have been found in *B. subtilis* (Okubo & Yanagida, 1968; Georgopoulos, 1969) and it seems likely that they will be found in other differentiating bacteria.

It has been repeatedly mentioned that the nature of the primary lesion in differentiation mutants is often not known. Indeed it is not known if the lesions lie in structural genes. When a mutation that blocks differentiation is suppressed by a nonsense suppressor, this establishes that the mutant gene codes for a protein. This analysis was applied by Hoch & Spizizen (1969) to *spo* loci of *B. subtilis*, and they found that some

spoOA mutations were suppressible. The *spoOA* locus is highly pleiotropic and is associated with the start of sporulation (Table 2). That it codes for a protein suggests some sort of positive control over subsequent events (Hoch & Spizizen, 1969).

The identification of suppressible differentiation mutations is clearly useful in the above context, although it has not so far been exploited extensively. The identification of nonsense mutations acquires greatly enhanced importance when combined with cloning techniques. Comparison of the protein product of a cloned gene with the product of the cloned gene bearing a nonsense mutation provides a clean way for identifying the protein product of that gene.

It has been argued elsewhere that mutants with temperature-sensitive nonsense suppressors could add considerably to studies of differentiation (Piggot & Coote, 1976). They could make possible identification of the time of transcription of genes for differentiation, and they might make new classes of conditional mutant available. Unfortunately little progress has been made in this direction.

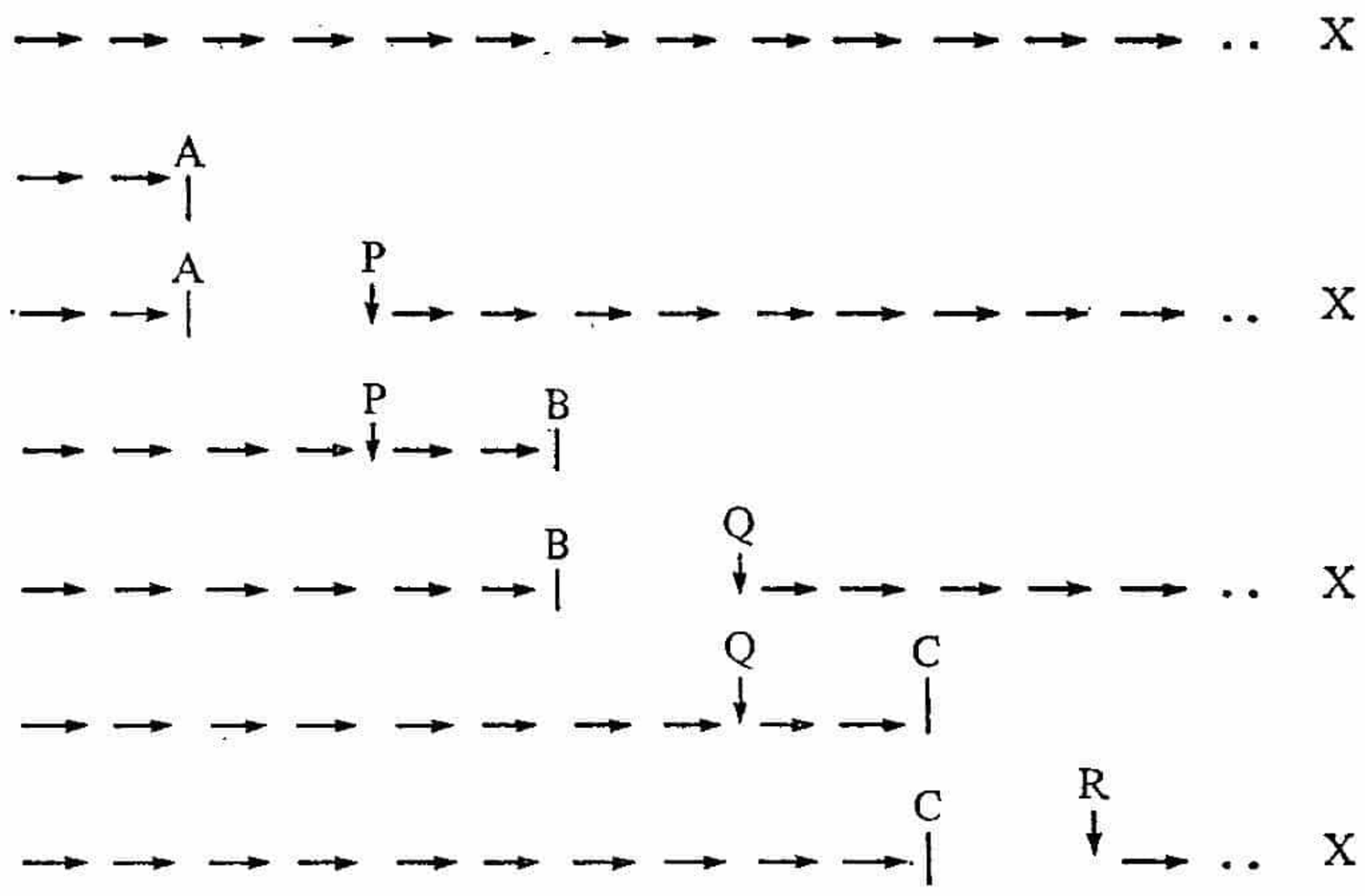

Fig. 1. 'Leap-frog' analysis of the sequence of gene expression leading to an event X that is part of (or close to) the sequence of events during differentiation. The mutations A, B and C prevent event X whereas mutations P, Q and R restore event X even though there are mutations earlier in the sequence. Arrows indicate a functioning sequence.

(*b*) *Partial suppressors.* Many mutations blocking differentiation are highly pleiotropic, causing the mutants to lose a number of properties associated with the later stages of differentiation. It is possible to isolate partial revertants of the mutants that have regained some, but not necessarily all, of the wild-type properties. This phenomenon, termed partial suppression, should not be confused with nonsense suppression and may occur by a completely different mechanism. The pleiotropy of the original mutation indicates that differentiation proceeds by a dependent sequence of events. The partial suppressor mutation has somehow disrupted the control of this sequence.

Partial suppressors can be studied in two ways: (*a*) to try to work out the sequence of genes involved in the dependent sequence of events; (*b*) to try to understand the nature of the control of that sequence. The first use, by a method which can be termed 'leap-frogging', is illustrated in Fig. 1. The starting strain bears a mutation, A, that

blocks differentiation, and in particular, prevents event X that is associated with a late stage of differentiation. A partial suppressor mutation, P, that restores event X is then induced. Depending on the complexity of the differentiation, and the nature of A, P, and X, the ability to differentiate may or may not be restored; no assumption about this is made here. Starting with a strain bearing mutation P, a mutation B that prevents expression of event X is then induced. Starting with a strain bearing mutation B, a mutation Q, that restores expression of event X is induced etc. From this a sequence of gene expression leading to event X can be deduced:

$$A \longrightarrow P \longrightarrow B \longrightarrow Q \longrightarrow C \longrightarrow R \longrightarrow X.$$

Several variations of the 'leap-frog' approach could be imagined. The rationale of the approach is not upset by the sequence deduced being part of a much more complex system involving parallel and interacting sequences. It adds an additional technique to those described previously for ordering events during differentiation. Needless to say the actual experiments have not reached the complexity of even the hypothetical scheme, although a start has been made for *B. subtilis* using *spo* mutations and production of alkaline phosphatase as the late event (Piggot & Taylor, 1977).

Two years ago there was no positive evidence of partial suppressors helping to identify the nature of the controls involved in endospore formation (Piggot & Coote, 1976). Most thoroughly investigated was a series of mutations that overcame some of the effects of *spoO* mutations (Guespin-Michel, 1971*a*, *b*; Ito, Mildner & Spizizen, 1971; Ito, 1973). None of the mutations restored the ability to sporulate. The mutations situated in one locus in particular, *cpsX*, presented a complex picture: different mutations suppressed to different extents, and the extent of suppression was also dependent on the *spoO* locus (Guespin-Michel, 1971*b*).

Hoch and collaborators have recently studied similar types of mutation, which they have called *abr*. Mutations in the *abrB* locus, which appears to be similar to *cpsX*, were associated with changes in the pattern of 50S ribosomal proteins seen on 2-dimensional polyacrylamide gels (Trowsdale, Chen & Hoch, 1978; Shiflett & Hoch, 1978). The result is suggestive of translational control. There is still no unequivocal explanation as to why ribosomal mutations should revert *spo* mutants for the particular characters used (antibiotic sensitivity, protease formation, antibiotic formation, competence for transformation, phage sensitivity), nor as to why some ribosomal proteins can be changed, or apparently lost, without affecting other properties of the bacteria. However, the result gives a frame of reference for theories, and a guide for further experiments.

(v) *Cell–cell interactions*

There are as yet few genetic experiments with bacteria that could be said to relate to this fascinating topic. Two sets of experiments will be discussed, one for *Myxococcus xanthus*, the other for *B. subtilis*. Although both probably relate to cell–cell interaction, they do so in different ways and are quite dissimilar. It is not yet clear that the methods described will be widely applicable.

(*a*) *Cell–cell interaction in the movement of* Myxococcus xanthus. *Myxococcus xanthus*

stands somewhat apart from the other species discussed in that the cells can aggregate to form a multi-cellular fruiting body. The initial part of this process is migration by a gliding movement. The system has only recently been studied genetically, but Hodgkin & Kaiser (1977) have already produced a very interesting analysis of motility mutants. Immobile mutants could be grouped in six classes. Mutants in class A could not be stimulated to become motile. Mutants in classes B to F could be stimulated to become motile by close contact with another mutant from any other class (including A). Mutations in any one of 17 loci gave the class A phenotype whilst classes B to F were each identified by mutations in a single locus. The most piquant, although not the only, interpretation suggested by the authors was that some cell to cell signal was required for the gliding motility; mutants in classes B to F were defective in some aspect of emission, whereas those in class A were defective in reception. Hodgkin & Kaiser made the interesting comment that the mutants they had studied were defective in the movement of individual cells and that there was genetic evidence for a separate system controlling movement of groups of cells. Even though there is still some doubt about the necessity of cell–cell interaction for motility of individual cells, this doubt can hardly exist for the movement of cells as groups.

(*b*) *Gene expression in mother cell and prespore of* B. subtilis. During endospore formation, bacterial cells undergo an unequal division into a large and a small cell, each containing a copy of the chromosome. The small cell, the prespore, is then engulfed by the larger cell, the mother cell. The prespore matures into the heat-resistant endospore, and is released by lysis of the mother cell. DNA of both mother cell and prespore is transcribed into RNA (Ryter, Bloom & Aubert, 1966).

Asporogenous (*spo*) mutants are unable to complete the process. By transforming them with wild-type DNA before the start of spore formation, it is possible to correct the defect. When this transformation is performed at the start of sporulation, it is possible that only one of the two copies of the mutant chromosome is transformed. This could segregate into either the mother cell or the prespore. If the wild-type chromosome segregates into the mother cell and this is all that is needed to form a spore, then the heat-resistant spore formed will still be genetically asporogenous. On the other hand, if the wild-type chromosome segregates into the prespore, and this is required to form a spore, then the heat-resistant spore will be genetically *spo*$^+$. From an analysis of this sort it is becoming clear that some spore loci need only be expressed in the mother cell whereas others must be expressed in the prespore (Piggot, 1978; Piggot & Lencastre, 1978, and unpublished observations). Thus some loci are expressed in one cell (the mother cell) so that another adjacent cell (the prespore) can become a spore.

IV. CONCLUSIONS

Only a few species of differentiating bacteria have been studied genetically. It is possible to talk in generalities about differentiation in these species and it is also possible to consider parts of the process in relatively precise terms. As soon as one attempts to discuss general schemes in precise terms, problems arise. However, these problems are often interesting and suggestive of further experiments. One example

will suffice as it concerns a, if not the, central feature of all the systems that have been extensively analysed, namely the essentially dependent sequence of events. The evidence for it is very clear – differentiation mutations cause differentiation-associated events to be lost in a characteristic and ordered manner (Table 2). A likely sequence of gene expression can be worked out from studies of double mutants, and of reciprocal shift, and by leap-frogging. It is already clear that there are parallel and interacting sequences rather than a single dependent sequence. In general terms, it is also clear that controls are operated at transcription of DNA into RNA, at translation of RNA into protein, and post-translationally. However, it is never clear which of these is operating at any precise point in the dependent sequence of events; the term 'event' must at present be kept very vague. If we consider only sequential induction of gene transcription (and ignore also switching-off) it is possible to build more detailed models (Halvorson, 1965; Mandelstam, 1976). This is illustrated in Fig. 2 in which the

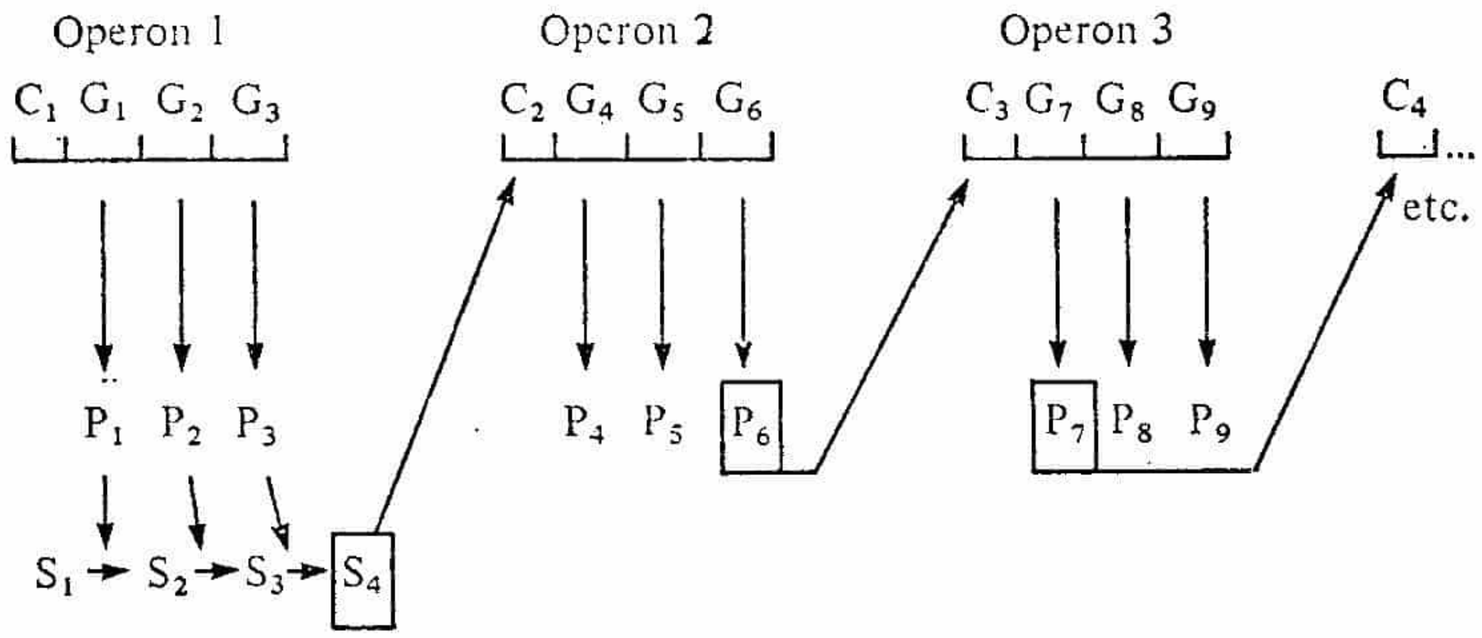

Fig. 2. Each operon is indicated as containing several genes (G_1, G_2, etc.); it is unlikely that an operon in a dependent sequence would contain a single gene unless that gene could control two or more functions. Each operon also contains an element (C_1, etc.) controlling operon transcription. The protein products of the genes are indicated as P_1, P_2, etc. Where those proteins are enzymes they act on a substrate S, etc. (e.g. P_1 on S_1, P_2 on S_2). Moieties S_4, P_6 and P_7 act on control elements C_2, C_3 and C_4 respectively to switch on transcription of the operon in question.

activation of a series of operons in a dependent sequence is considered. The switch-on of successive operons (via elements C_1, C_2, C_3) could presumably be mediated by low molecular weight products of enzyme action (e.g. S_4 in Fig. 2) or by proteins (e.g. P_6 or P_7, Fig. 2). Differing consequences of single-point mutations would be anticipated. Mutation in G_1, G_2, and G_3 would all prevent expression of subsequent operons, and would be difficult to distinguish unless P_1, P_2 and P_3 (or S_1, S_2, S_3 or S_4) were identifiable. In operon 2, mutation in G_6 would prevent expression of subsequent operons, whereas mutations in G_4 and G_5 would not (unless polar); thus mutations located very close together could give rise to very different phenotypes, as mutations in G_4 and G_5 would have much less pleiotropic effects than mutation in G_6, and might even allow complete differentiation in a way that was barely distinguishable from the wild-type. Mutation in a control element such as C_3 could prevent the operon being switched on. The phenotype of such a mutation in C_3 might be virtually indistinguishable from that

of a mutation in G_6 (in a different operon). Alternatively, mutation in C_3 could cause operon 3 to function independently of operon 2, in which case the phenotype might be that of the partial suppressors discussed earlier. Such mutations could map very close to mutations giving rise to very different phenotypes.

The detailed analysis implied in the above discussion may soon be possible by a combination of classic genetics and the methods of genetic engineering. The knowledge of the organization and control of gene expression gained by genetic methods should provide the necessary framework. Good biochemistry and good molecular biology should be able to effect a comprehensive analysis on this framework.

V. SUMMARY

1. Bacterial cell differentiation has mainly been studied genetically in four species, in each of which systems of genetic exchange have been described: *Bacillus subtilis*, *Streptomyces coelicolor*, *Myxococcus xanthus*, and *Caulobacter crescentus*. In the first three species the process is a response to starvation and leads to a resting cell (spore). In *C. crescentus* the vegetative cell cycle itself involves a process of differentiation.

2. Much information can be gained without genetic exchange. Studies of mutants and of different species can be used to determine the relevance of biochemical and morphological changes during differentiation to the process of differentiation.

3. Single-point mutations that block differentiation generally have several effects on differentiation-associated characters. When different mutants are compared, the corresponding characters are seen to be lost in an ordered fashion. This indicates that differentiation involves a dependent sequence of events in which occurrence of any event is dependent upon successful occurrence of earlier events. Dependent sequences have been found for all systems so far analysed in this way.

4. Dependent sequences can be controlled at the transcription of DNA into RNA, at the translation of RNA into protein, and post-translationally during the assembly of structures. Sequential induction of operons provides a useful model for transcriptional control. Studies of temperature-sensitive mutants indicate that the system is both complex and delicate.

5. Further methods of genetic analysis depend on genetic exchange. The introduction of cell fusion and of gene cloning may allow a wider range of species to be studied.

6. In systems allowing genetic exchange it is possible to estimate the number of genetic loci specifically involved in differentiation. Expression of some 40 to 50 *spo* loci is required for spore formation by *Bacillus subtilis*.

7. Studies of (mero) diploids will be of limited value until much more is known about the primary products, and the fine structure, of the genetic loci involved in differentiation. Such knowledge may come from genetic engineering.

8. Several methods have been used to decide if events are in a dependent sequence. They can also be used to order the events in a dependent sequence.

9. Detailed examination of the implications of a dependent sequence of operon transcription leads to precise and testable predictions.

10. The movement of *Myxococcus xanthus* provides a system for genetic analysis of cell–cell interaction.

11. Sporulation of *Bacillus subtilis* requires interaction of the mother cell and the prespore, and it is now possible to identify which *spo* loci are expressed in the two cell types.

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